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(54) Title: A cDNA AND PEPTIDE WITH RELATION TO CANCER AND WEIGHT LOSS			
(57) Abstract <p>Novel isolated cancer cachectic factor peptides (CCF) and an isolated precursor form hereof (preCCF) is provided. The invention further provides DNA constructs encoding cancer cachectic factors, and DNA constructs encoding precursors of cancer cachectic factors. The invention further relates to recombinant vectors, and recombinant host cells comprising said DNA constructs. Furthermore methods of producing said CCF peptides or said preCCF polypeptide are provided. In view of cachexia being one of the most common adverse effects of malignancy occurring in about one half of untreated cancer patients, and thus being responsible for both shorter survival times and a decreased response to therapy, there is a need in the art for agents that regulates this unwanted loss of tissue. It is an object of the present invention to provide such agents. It is a further object of the invention to provide medicaments and methods for preventing or treating conditions or disorders arising from obesity, NIDDM, or Syndrome X.</p>			

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TITLE

A cDNA AND PEPTIDE WITH RELATION TO CANCER AND WEIGHT LOSS.

5 FIELD OF INVENTION

The present invention relates to glycosylated or non-glycosylated peptides with cachectic effects (cancer cachectic factor, CCF), a precursor form thereof (preCCF), DNA constructs encoding said peptide or precursor, a method of producing said glycosylated or
10 non-glycosylated peptide or precursor, and methods to stimulate or prevent the native synthesis or action of said peptide or precursor. The invention further relates to recombinant vectors, recombinant host cells, pharmaceutical compositions, use for the preparation of medicaments, and methods of treatment.

15

BACKGROUND OF THE INVENTION

Cachexia is a metabolic state characterized by dramatic weight loss due to depletion of both muscle and adipose tissue. Many diseases, such as bacterial or parasitic infectious
20 diseases, multiple organ traumas and cancer, are often accompanied by cachexia.

Background art

Recently, a cancer cachectic factor from a mouse bearing the cachexia-inducing adenocarcinoma MAC16 was described in the literature (Todorov et al (1996) Nature
25 379: 739-742). This factor, which was purified from isolated MAC16 solid tumours, is a proteoglycan with a MW of 24kD containing a small peptide core of about 20 amino acids. Injection of purified factor into mice gave rise to weight loss, due both to depletion of muscle and adipose tissue. No effect on food and water intake was observed. In an *in vitro* assay using isolated adipocytes, the factor led to increased lipolysis (McDevitt et al.
30 (1995) Cancer Res. 55: 1458-1463), and in a similar assay using isolated muscle tissue increased proteolysis was observed; the latter effect was shown to be abolished by prior *in vitro* treatment of the factor with enzymes that digest glycosyl side chains (Todorov et al (1996) Cancer Res 56: 1256-1261). Incubation of the factor with a monoclonal antibody raised against the factor also abolished the *in vitro* proteolysis promoting effect
35 of the factor, whereas the *in vitro* lipolysis effect was not affected. However, the

antibody was shown to prevent factor-induced weight loss when mice were injected with this antibody prior to injection with factor. The monoclonal antibody used was shown to react with an epitope on the glycan part of the factor.

- 5 The N-terminal sequence of the murine peptide core was determined as Tyr-Asp-Pro-Glu-Ala-Ala-Ser-Ala-Pro-Gly-Ser-Gly-Asp-Pro-Ser-His-Glu-Ala-(Ser)-(Ala). A similar factor was purified from the urine of human patients with various strongly cachectic tumours. N-terminal sequence analysis performed on the peptide core of the human factor yielded 14 amino acids which were identical to the mouse sequence. Because of the high
10 degeneracy of many of the codons for the amino acids in the core peptide the amino acid sequence could not be used to characterize or isolate either the mouse or the human cDNA or gene encoding the peptide. Thus, the genetic and molecular origin of the factor could not be determined, i.e. it was not determined whether the factor was synthesised by specific tumour cells, nor was it determined whether it was made from a larger precursor.
15 A synthetic 20 amino acid long peptide with the sequence shown above had no effect *in vivo*.

- The mechanism of action of CCF is completely unknown. One possibility is that it acts via binding to cell surface receptors, thereby activating some as yet unidentified
20 intracellular pathway. However, although iodinated purified factor was shown to bind to muscle cells (Todorov et al., op. cit.) a specific receptor interaction was not proven.

- US Patents Nos. 5,3885,740 and 5,219,579 (Tisdale et al.) describes a biologically active lipolytic factor having an average molecular weight substantially less than 5000 daltons
25 and comprising at least one active molecular species having a molecular weight of about 1500 daltons. The factor was isolated from the MAC16 cachexia-inducing tumour described above. The molecular nature of this factor was not disclosed. Thus, it is unknown whether it is related to the 24 kD cancer cachectic factor described above.

- 30 In view of cachexia being one of the most common adverse effects of malignancy occurring in about one half of untreated cancer patients, and thus being responsible for both shorter survival times and a decreased response to therapy, there is a need in the art for agents that regulates this unwanted loss of tissue. It is an object of the present invention to provide such agents. It is a further object of the invention to provide
35 medicaments and methods for preventing or treating conditions or disorders arising from

obesity, NIDDM, or Syndrome X.

SUMMARY OF THE INVENTION

5

The present invention provides isolated polynucleotide molecules encoding a cancer cachectic factor peptide (CCF) and fragments, variants and homologues hereof. Within a preferred embodiment hereof, the encoded peptide is a human peptide. Within one aspect, the isolated polynucleotide molecule of the present invention is selected from the group consisting of (a) a DNA molecule having a coding sequence corresponding to SEQ ID NO:15; (b) a DNA sequence which hybridizes to the DNA sequence shown in SEQ ID NO:15; (c) or a DNA sequence which has a sequence homology of at least 60% to the DNA sequence shown in SEQ ID NO:15. Within another aspect, the isolated polynucleotide molecule of the present invention is selected from the group consisting of (a) a DNA molecule having a coding sequence corresponding to SEQ ID NO:16; (b) a DNA sequence which hybridizes to the DNA sequence shown in SEQ ID NO:16; (c) or a DNA sequence which has a sequence homology of at least 60% to the DNA sequence shown in SEQ ID NO:16.

10 20 The invention further provides isolated polynucleotide molecules encoding a precursor (preCCF) of a cancer cachectic factor peptide, and fragments, variants and homologues hereof.

Within one aspect, the isolated polynucleotide molecule is selected from the group consisting of (a) a DNA molecule having a coding sequence corresponding to SEQ ID NO:1; (b) a DNA sequence which hybridizes to the DNA sequence shown in SEQ ID NO:1; (c) or a DNA sequence which has a sequence homology of at least 60% to the DNA sequence shown in SEQ ID NO:1.

25 30 A related aspect of the invention provides isolated cancer cachectic factor peptides. In a preferred aspect, the peptide has an amino acid sequence as shown in SEQ ID NO:17. In another preferred aspect, the peptide has an amino acid sequence as shown in SEQ ID NO:18. In a preferred embodiment, the isolated polypeptide is a human peptide.

35 The invention further provides isolated cancer cachectic factor precursor polypeptides. In a

preferred aspect, the precursor polypeptide has an amino acid sequence as shown in SEQ ID NO:2. In a preferred embodiment, the isolated precursor polypeptide is a human peptide.

- 5 The cancer cachectic factor peptides may be glycosylated or non-glycosylated. In a preferred embodiment, the peptide is O-glycosylated at one or more of the serine residues and/or N-glycosylated at the asparagine residues.

- Another aspect of the present invention provides methods of preparing cancer cachectic factor peptides or precursor forms hereof. Recombinant vectors and host cells for use in the preparation of said peptides are provided as well.
- 10

- The invention further provides a method of inducing weight loss, a method of treating conditions or disorders arising from obesity, methods of preventing or treating non-insulin dependent diabetes (NIDDM) and Syndrome X, and the use of a cancer cachectic factor peptide to prepare medicaments for pharmaceutical treatment, and pharmaceutical compositions useful for the treatment of said indications.
- 15

- The invention also provides methods for preventing unwanted endogenous synthesis or activity of the cancer cachectic factor, e.g. in cancer patients, thus preventing weight loss.
- 20

LIST OF FIGURES

- 25 Fig. 1 shows the cDNA insert of clone p24k-inc with translation in the frame containing the 20 amino acids search string (SEQ ID NO:1).
Fig. 2 shows the 110 amino acids precursor peptide (named preCCF) containing the 20 amino acids search string (SEQ ID NO:2).
Fig. 3a shows the 24 aa cancer cachectic peptide (SEQ ID NO:17).
30 Fig. 3b shows the 20 aa cancer cachectic peptide.(SEQ ID NO:18).
Fig. 4 shows a Southern blot of preCCF expressed in an insect cell line.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery that a cDNA clone isolated from a human breast tumour library contains a coding region with extensive homology to a 20 amino acid sequence obtained from the peptide core of a mouse tumor derived proteoglycan with cachectic effects (Todorov et al, op.cit). Characterization of the cDNA clone showed it to encode a 110aa polypeptide called preCCF shown in SEQ ID 2. This 110aa polypeptide has the characteristics of a secretory peptide, with amino acid 1 to 18 constituting a potential signal sequence. Signal sequences acts to direct proteins into the secretory pathway of cells, where several posttranslational modifications are known to occur, such as proteolytic processing and glycosylation (Darnell, Lodish and Baltimore (1986) Molecular Cell Biology, Scientific American Books Inc., p.940-964).

In the secretory pathway proteolytic processing of the preCCF signal sequence by a Signal Peptidase (Dalbey and von Heijne (1992) TIBS 17, p.474-478) is expected to generate a 92aa peptide with an N-terminal A (A-19 of SEQ ID 2). PreCCF contains several other potential sites for proteolytic processing, eg. several residues of the basic amino acids K and R which, either alone or in pairs, often constitute processing sites for proteases. The Prohormone Convertase (PC) family (Rouille et al (1995) Frontiers in Neuroendocrinology 16 (4) p. 322-361) is an example of such proteases that are located in the secretory pathway. PC processing after K-42 of SEQ ID 2, combined with signal peptidase cleavage after C-18 of SEQ ID 2 would generate a 24aa peptide with the sequence shown in SEQ ID 17. Stepwise processing, catalyzed by Exopeptidases, of the 24aa peptide could generate shortened versions, such as a 20aa peptide. Endo- and exopeptidases with the required specificities may be found in the extracellular space as well as in the secretory pathway, thus one or more of the described processing steps could potentially occur after secretion of a precursor form from the cell. The 20aa form which would be generated from the 24aa form disclosed in this application is shown in SEQ ID 18. It should be noted that this sequence differs in two positions from that given by Todorov et al (op.cit): Residues 13 and 15 are N and C, respectively, in SEQ ID 18, but D and S in Todorov et al. Todorov et al reported that the obtained sequence of a human cachectic factor with immunological similarities to the mouse cachectic factor was identical to the mouse sequence, but only 14 residues were determined of the human factor. Thus, the residue 15 difference may represent a species difference between mouse and man; alternatively, it might represent an amino acid sequencing error, since S and C

are known to give ambiguous signals in the sequencing reaction. We believe that the difference for residue 13 must be due to an amino acid sequencing error, since it is well known that amino acid sequencing often mistakes D for N due to chemical deamidation during the sequencing reactions.

5

Well-known glycosylation reactions that can occur on proteins in the secretory pathway are also relevant for the present invention. PreCCF contains two potential sites for N-linked glycosylation on asparagine (N-32 and N-44 shown in SEQ ID 2 with #), although none of the sequence contexts are optimal for glycosylation (Schacter (1994) Tools For Glycobiology, Oxford GlycoSystems, p.9-11). There are several potential sites for O-linked glycosylation on Serine (shown with * in SEQ ID 2). Addition of N- and O-linked glycosyl side chains would result in a proteoglycan with a molecular weight substantially larger than that of the polypeptide, with the actual molecular weight depending on the number and sizes of the glycan chains added. The 20aa peptide shown in SEQ ID 18 contains 3 potential sites for O-glycosylation and one for N-glycosylation. Glycosylation of one or more of these sites could result in the formation of a 24kD proteoglycan. In addition to the glycosylation that may occur by enzymatic means in the secretory pathway, glycosylation can also occur by non-enzymatic means in the extra-cellular space.

20

Thus, it is clear that the preCCF sequence disclosed in this application by known biological processes can give rise to the 24K cachectic factor described by Todorov et al, and that it thus represents a precursor to this factor. The characterization of the human preCCF coding sequence has revealed two amino acid differences between this sequence and that disclosed for mouse in Todorov et al. These two differences may be important for biological effect in humans. Further, we have shown that a likely primary processing product of preCCF is a 24aa peptide, which may have similar or more potent effects than the 20aa peptide. Knowledge of the correct human sequence allows for manufacture of the factor by heterologous expression by various means and for various purposes, as detailed below. Further, knowledge of the preCCF cDNA and its encoding gene allows for development of substances that can up- or down-regulate CCF activity *in vivo*, with important pharmaceutical and medical implications, as also detailed below.

Throughout this application, the following standard abbreviations are used to indicate specific nucleotide bases:

35

C = cytosine A = adenine T = thymine G = guanine

Throughout this application, the following standard one-letter abbreviations are used to indicate specific amino acids:

A = Ala	R = Arg	N = Asn	D = Asp	C = Cys
Q = Gln	E = Glu	G = Gly	H = His	I = Ile
L = Leu	K = Lys	M = Met	F = Phe	P = Pro
S = Ser	T = Thr	W = Trp	Y = Tyr	V = Val

The polypeptide

In the present context, the term "peptide" is understood to include the mature cancer cachectic factor peptide or a precursor form thereof. The term "peptide" is also understood to include glycosylated forms of the CCF peptide, precursor or functional fragments. Furthermore, the term "peptide" is intended to include homologues of said peptide. Such homologues comprise an amino acid sequence exhibiting a degree of identity of at least 50 %, such as at least 75 %, and more particularly at least 90% identity with the amino acid sequence of human CCF peptide. The degree of identity may be determined by conventional methods, see for instance, Altshul et al., Bull. Math. Bio. 48: 603-616, 1986, and Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89: 10915-10919, 1992. Alternatively, the homologue of the peptide may be one encoded by a nucleotide sequence hybridizing with an oligonucleotide probe prepared on the basis of the nucleotide sequence shown in SEQ ID NO:1 or the amino acid sequence shown in SEQ ID NO:2 under high stringency conditions (i.e. pre-soaking in 5X SSC and pre-hybridizing for 1 hr. at about 40°C in a solution of 20% formamide, 5X Denhardt's solution, 50 mM sodium phosphate, pH 6.8, and 50 mg denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with the labelled oligonucleotide probe for 18 hrs. at about 40°C, followed by a wash in 0.4X SSC at a temperature of about 45°C).

Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using standard detection procedures (e.g. Southern blotting).

Homologues of the present peptide may have one or more amino acid substitutions, deletions or additions. These changes may be of a minor nature, that is conservative

amino acid substitutions that do not significantly affect the folding or activity of the peptide, small deletions, typically of one to about five amino acids, small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 15 residues, or a small extension that facilitates purification, such as a poly-histidine tract, an antigenic epitope or a binding domain. See in general Ford et al., Protein Expression and Purification 2: 95-107, 1991. Examples of conservative substitutions are within the group of basic amino acids (such as arginine, lysine, histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine, valine), aromatic amino acids (such as phenylalanine, tryptophan, tyrosine) and small amino acids (such as glycine, alanine, serine, threonine, methionine).

It will be apparent to persons skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acids essential to the activity of the polypeptide of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244, 1081-1085, 1989). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (e.g. *in vivo*: weight loss, *in vitro*: proteolysis or lipolysis, which, for example, can be determined as described by Todorov et al., (1996) Cancer Res 56: 1256-1261) in order to identify amino acid residues that are critical to the activity of the molecule.

The homologue may be an allelic variant, i.e. an alternative form of a gene that arises through mutation, or an altered peptide encoded by the mutated gene, but having substantially the same activity as the native peptide. Hence mutations can be silent (no change in the encoded peptide) or may encode peptides having altered amino acid sequence.

30

The homologue of the present CCF peptide may also be a species homologue, i.e. a peptide with a similar activity derived from another species. Examples of species homologues of the peptide are human, bovine, rat, hamster, guinea pig and porcine versions.

35

A homologue of the polypeptide may be isolated by preparing a genomic or cDNA library of a cell of the species in question, and screening for DNA sequences coding for all or part of the homologue by using synthetic oligonucleotide probes in accordance with standard techniques, e.g. as described by Sambrook et al., Molecular Cloning: A

- 5 Laboratory Manual, 2nd. Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989, or by means of polymerase chain reaction (PCR) using specific primers as described by Sambrook et al., supra.

- Further homologues of the present CCF peptide are those which are immunologically
10 cross-reactive with antibodies raised against the peptide or polypeptide of the invention. Cross-reactivity may, for example, be determined by Western analysis (see, for example, Current Protocols in Molecular Biology on CD-ROM, eds. Ausubel et al, 1996, unit 10.8)

- 15 The CCF peptide or preCCF of the invention is in isolated form, i.e. found in a condition other than its native environment, such as apart from peptides, proteins, or other organic residues originating from the used expression systems e.g. insect cells, mammal cells or plant cells.
- 20 The currently preferred polypeptides of the invention is the ones comprising the amino acid sequence shown in SEQ ID NO:2, SEQ ID NO:17, or SEQ ID NO:18.

- The peptide as specified above may be made by recombinant DNA techniques followed by protein purification and processing procedures in accordance with procedures well
25 established in the art. These include in vivo synthesis of a precursor form of the peptide, followed by purification and in vitro processing to the desired peptide form.

- A plasmid p24k-inc comprising the cDNA according to the present invention (pND-N6) as well as an *E.coli* transformant containing this plasmid (ND-N6) were deposited on 27
30 August 1996 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. The transformant was accorded DSM Accession No. DSM 11126, and the plasmid was accorded DSM Accession No. DSM 11127.

DNA constructs

A DNA sequence encoding the CCF or preCCF peptide may be isolated or synthesized on the basis of the sequence disclosed in this application, for instance obtained by
5 preparing a genomic or cDNA library from an appropriate tissue and screening for DNA sequences coding for all or part of the CCF precursor peptide by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., *supra*). For the present purpose, the DNA sequence encoding the peptide is preferably of human origin.

10

The DNA construct encoding the polypeptide may also be prepared synthetically by established standard methods, e.g. the phosphoramidite method described by Beaucage and Caruthers, *Tetrahedron Letters* 22 (1981), 1859-1869, or the method described by Matthes et al., *EMBO Journal* 3 (1984), 801-805. According to the phosphoramidite
15 method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

Furthermore, the DNA construct may be of mixed synthetic and genomic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments
20 of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire DNA construct, in accordance with standard techniques.

The DNA construct may also be prepared by polymerase chain reaction using specific primers, for instance as described in US 4,683,202 or Saiki et al., *Science* 239 (1988),
25 487 - 491, or Sambrook et al., *supra*.

In a currently preferred embodiment, the DNA construct comprises the DNA sequence shown in Fig. No. 1 in this application as well as nucleic acid sequences encoding human preCCF, but which differ from the DNA sequence shown in Fig. No. 1 by virtue of the
30 degeneracy of the genetic code. The DNA construct further includes nucleic acid sequences which hybridize to a nucleic acid molecule (either genomic, synthetic or cDNA or RNA) encoding the human peptide under the conditions of medium stringency (i.e. pre-soaking in 5X SSC and pre-hybridizing for 1 hr. at about 40°C in a solution of 20% formamide, 5X Denhardt's solution, 50 mM sodium phosphate, pH 6.8, and 50 mg
35 denatured sonicated calf thymus DNA, followed by hybridization in the same solution

supplemented with the labelled oligonucleotide probe for 18 hrs. at about 40°C, followed by a wash in 0.4X SSC at a temperature of about 35°C). This could, for instance, be DNA sequences encoding preCCF from other species, e.g. rat, bovine, hamster, guinea pig or porcine preCCF.

5

Recombinant vectors

To express CCF or preCCF peptide, the DNA construct encoding the peptide is inserted into an appropriate recombinant vector. This may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend
10 on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into
15 which it has been integrated.

The vector is preferably an expression vector in which the DNA sequence encoding the CCF peptide or preCCF peptide is operationally linked to additional segments required for transcription of the DNA. In general, the expression vector is derived from plasmid
20 or viral DNA, or may contain elements of both. The term, "operationally linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates at a promoter and proceeds through the DNA sequence coding for the peptide and stops at a terminator.

25 The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the DNA encoding the
30 CCF or preCCF peptide in mammalian cells are the SV40 promoter (Subramani et al., Mol. Cell Biol. 1 (1981), 854 -864), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science 222 (1983), 809 - 814) or the adenovirus 2 major late promoter.

An example of a suitable promoter for use in insect cells is the polyhedrin promoter (US
35 4,745,051; Vasuvedan et al., FEBS Lett. 311, (1992) 7 - 11), the P10 promoter (J.M.

Vlak et al., J. Gen. Virology 69, 1988, pp. 765-776), the *Autographa californica* polyhedrosis virus basic protein promoter (EP 397 485), the baculovirus immediate early gene 1 promoter (US 5,155,037; US 5,162,222), or the baculovirus 39K delayed-early gene promoter (US 5,155,037; US 5,162,222).

5

An example of a suitable promoter for use in plant cells is the CaMV promoter (Cauliflower Mosaic Virus). An example of a suitable vector for use in a plant expression system is *Agrobacterium tumefaciens* TI plasmid. (Magnuson et al. (1996), Protein Expression and purification 7, p.220-228)

10

Examples of suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255 (1980), 12073 - 12080; Alber and Kawasaki, J. Mol. Appl. Gen. 1 (1982), 419 - 434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al, eds.), Plenum Press, New York, 1982), or the TPI1 (US 4,599,311) or ADH2-4c (Russell et al., Nature 304 (1983), 652 - 654) promoters.

Examples of suitable promoters for use in bacterial host cells include the promoter of the *Bacillus stearothermophilus* maltogenic amylase gene, the *Bacillus licheniformis* alpha-amylase gene, the *Bacillus amyloliquefaciens* BAN amylase gene, the *Bacillus subtilis* alkaline protease gene, or the *Bacillus pumilus* xylosidase gene, or by the phage Lambda P_R or P_L promoters or the E. coli lac, trp or tac promoters.

The DNA sequence encoding the peptide may also, if necessary, be operationally connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.) or (for fungal hosts) the TPI1 (Alber and Kawasaki, op. cit.) or ADH3 (McKnight et al., op. cit.) terminators. The vector may further comprise elements such as polyadenylation signals (e.g. from SV40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication.

When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2m replication genes REP 1-3 and origin of replication.

- 5 The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P.R. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For
10 filamentous fungi, selectable markers include amdS, pyrG, argB, niaD, sC.

- The preCCF peptide contains a natural signal sequence which can direct the peptide into the secretory pathway of the host cells, particularly if the host cell is mammalian. When routed through the secretory pathway of a eucaryotic cell the peptide may become
15 glycosylated, which may be advantageous for some embodiments of the invention. It may be advantageous, particularly when using other host cells than mammalian cells, to replace this signal sequence with another secretory signal sequence (also known as a leader sequence, preprosequence or presequence) provided by the recombinant vector. The secretory signal sequence is joined to the DNA sequence encoding the desired
20 peptide fragment in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the peptide. The secretory signal sequence may be from a gene encoding another secreted protein, preferably from a protein well secreted from the chosen host cell.
- 25 For secretion from insect cells, the signal peptide may conveniently be derived from a secreted insect cell protein (cf. WO 90/05783), such as the lepidopteran *Manduca sexta* adipokinetic hormone precursor signal peptide (cf. US 5,023,328).

- For secretion from yeast cells, the secretory signal sequence may encode any signal
30 peptide which ensures efficient direction of the expressed peptide into the secretory pathway of the cell. The signal peptide may be a naturally occurring signal peptide, or a functional part thereof, or it may be a synthetic peptide. Suitable signal peptides have been found to be the alpha-factor signal peptide (cf. US 4,870,008), the signal peptide of mouse salivary gland amylase (cf. O. Hagenbuchle et al., Nature 289, 1981, pp. 643-
35 646), a modified carboxypeptidase signal peptide (cf. L.A. Valls et al., Cell 48, 1987,

pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), or the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., Yeast 6, 1990, pp. 127-137).

- 5 For efficient secretion in yeast, a sequence encoding a leader peptide may also be inserted downstream of the signal sequence and upstream of the DNA sequence encoding the CCF or preCCF peptide. The function of the leader peptide is to allow the expressed peptide to be directed from the endoplasmic reticulum to the Golgi apparatus and further to a secretory vesicle for secretion into the culture medium (i.e. export of the peptide across
10 the cell wall or at least through the cellular membrane into the periplasmic space of the yeast cell). The leader peptide may be the yeast alpha-factor leader (the use of which is described in e.g. US 4,546,082, EP 16 201, EP 123 294, EP 123 544 and EP 163 529). Alternatively, the leader peptide may be a synthetic leader peptide, which is to say a leader peptide not found in nature. Synthetic leader peptides may, for instance, be
15 constructed as described in WO 89/02463 or WO 92/11378.

The procedures used to ligate the DNA sequences coding for the CCF or preCCF peptide, the promoter and optionally the terminator and/or secretory signal sequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook
20 et al., op.cit.).

- The DNA sequence encoding the CCF or preCCF peptide introduced into the host cell may be either homologous or heterologous to the host in question. If homologous to the host cell, i.e. produced by the host cell in nature, it will typically be operationally
25 connected to another promoter sequence or, if applicable, another secretory signal sequence and/or terminator sequence than in its native form. The term "homologous" is intended to include a cDNA sequence encoding a polypeptide native to the host organism in question. The term "heterologous" is intended to include a DNA sequence not expressed by the host cell in nature. Thus, the DNA sequence may be from another
30 organism, or it may be a synthetic sequence.

Host cells

- The host cell into which the DNA construct or the recombinant vector of the invention is introduced may be any cell which is capable of producing the present peptide and
35 includes bacteria, yeast, insect cells, plant cells and higher eukaryotic cells.

Examples of suitable mammalian cell lines are the COS (ATCC CRL 1650), BHK (ATCC CRL 1632, ATCC CCL 10), CHL (ATCC CCL39) or CHO (ATCC CCL 61) cell lines. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J. Mol. Biol. 159 (1982), 601 - 621; Southern and Berg, J. Mol. Appl. Genet. 1 (1982), 327 - 341; Loyter et al., Proc. Natl. Acad. Sci. USA 79 (1982), 422 - 426; Wigler et al., Cell 14 (1978), 725; Corsaro and Pearson, Somatic Cell Genetics 7 (1981), 603, Graham and van der Eb, Virology 52 (1973), 456; and Neumann et al., EMBO J. 1 (1982), 841 - 845.

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Transformation of insect cells and production of heterologous polypeptides therein may be performed as described in US 4,745,051; US 4,879,236; US 5,155,037; 5,162,222; EP 397,485) all of which are incorporated herein by reference. The insect cell line used as the host may suitably be a *Lepidoptera* cell line, such as *Spodoptera frugiperda* cells or *Trichoplusia ni* cells (cf. US 5,077,214). Culture conditions may suitably be as described in, for instance, WO 89/01029 or WO 89/01028, or any of the aforementioned references.

Transformation of plant cells and production of heterologous polypeptides therein may be performed as described in Magnuson et al. (1996), Protein Expression and Purification 7, p.220-228. The plant cell line used as the host may suitably be *Nicotiana tabacum* (NT-1) suspension cells (see, for example, Magnuson et al., above).

Examples of suitable yeast cells include cells of *Saccharomyces* spp. or *Schizosaccharomyces* spp., in particular strains of *Saccharomyces cerevisiae* or *Saccharomyces kluyveri*. Methods for transforming yeast cells with heterologous DNA and producing heterologous polypeptides therefrom are described, e.g. in US 4,599,311, US 4,931,373, US 4,870,008, 5,037,743, and US 4,845,075, all of which are hereby incorporated by reference. Transformed cells are selected by a phenotype determined by a selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient, e.g. leucine. A preferred vector for use in yeast is the POT1 vector disclosed in US 4,931,373. The DNA sequence encoding the CCF- or preCCF peptide may be preceded by a signal sequence and optionally a leader sequence, e.g. as described above. Further examples of suitable yeast cells are strains of *Kluyveromyces*, such as *K. lactis*, *Hansenula*, e.g. *H. polymorpha*, or *Pichia*, e.g. *P. pastoris* (cf. Gleeson et al., J.

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Gen. Microbiol. 132, 1986, pp. 3459-3465; US 4,882,279).

Examples of bacterial host cells which, on cultivation, are capable of producing the CCF or preCCF peptide are grampositive bacteria such as strains of *Bacillus*, such as strains of
5 *B. subtilis*, *B. licheniformis*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*,
B. amyloliquefaciens, *B. coagulans*, *B. circulans*, *B. lautus*, *B. megatherium* or *B.*
thuringiensis, or strains of *Streptomyces*, such as *S. lividans* or *S. murinus*, or
gramnegative bacteria such as *Echerichia coli*. The transformation of the bacteria may be
10 effected by protoplast transformation or by using competent cells in a manner known per
se (cf. Sambrook et al., supra).

When expressing the peptide in bacteria such as *E. coli*, the peptide may be retained in the cytoplasm, typically as insoluble granules (known as inclusion bodies), or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case,
15 the cells are lysed and the granules are recovered and denatured after which the peptide is refolded by diluting the denaturing agent. In the latter case, the peptide may be recovered from the periplasmic space by disrupting the cells, e.g. by sonication or osmotic shock, to release the contents of the periplasmic space and recovering the peptide.

20 The transformed or transfected host cell described above is then cultured in a suitable nutrient medium under conditions permitting the expression of the CCF or preCCF peptide, after which the resulting CCF or preCCF peptide (or related peptides) are recovered from the culture.

25 The medium used to culture the cells may be any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection). The CCF or preCCF peptide produced by the cells may then be recovered
30 from the culture medium by conventional procedures including separation of the host cells from the medium by centrifugation or filtration, precipitation of the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gelfiltration chromatography, or affinity chromatography.

Antibodies

- The polypeptides of the invention can also be used to prepare antibodies that specifically bind to CCF or preCCF peptides. Immunogens may be full-length or portions of the CCF
- 5 or preCCF molecules or may be combined with a carrier.

As used herein, the term "antibodies" includes polyclonal antibodies, monoclonal antibodies, antigen-binding fragments hereof such as F(ab')₂ and Fab fragments, and the like, including genetically engineered antibodies. Antibodies are defined to be

10 specifically binding if they bind to a CCF peptide with a K_a of greater than or equal to 10⁷/M. The affinity of an antibody can be readily determined by one of ordinary skill in the art (see, for example, Roit, Essential Immunology, fifth ed., Blackwell Scientific Publications, 1984).

- 15 Methods for preparing polyclonal and monoclonal antibodies are well known in the art (see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, second Ed., Cold Spring harbor, NY, 1989; and Hurrel, Ed., Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press, Inc., Boca Raton, FL, 1982). As would be evident to one of ordinary skill in the art, polyclonal antibodies can be
- 20 generated from a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, and rats. The immunogenicity of a peptide or polypeptide may be increased through the use of an adjuvant such as Freund's complete or incomplete adjuvant. A variety of assays known to those skilled in the art can be utilized to detect antibodies which specifically bind to polypeptides (see Harlow and Lane (Eds.),
- 25 Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1988).

Monoclonal antibodies may be obtained by well-established methods, e.g. as described in A. Johnstone and R. Thorpe, Immunochemistry in Practice, 2nd. Ed., Blackwell Scientific Publications, 1987, pp. 35-43.

- 30 Generally, monoclonal antibodies are produced by immunizing an animal with a biological specimen or other foreign substance, obtaining antibody-producing cells from the animal, and fusing the antibody-producing cells with strains of neoplastic cells, e.g. tumour cells, to produce hybridomas which are isolated and cultured as monoclones. The monoclonal hybridomas may either be cultured *in vitro* or may be grown *in vivo* as

tumours in a host animal. Because each antibody-producing cell line produces a single unique antibody, the monoclonal cultures of hybridomas each produce a homogenous antibody population which may be obtained either from the culture medium of hybridoma cultures grown *in vitro* or from the ascitic fluid, or serum of a tumour-bearing host
5 animal. Not all of the clones which result from fusion of neoplastic cells with antibody-producing cells are specific for the desired foreign substance or antigen, because many of the hybridomas will secrete antibodies which the animal's immune system has generated in reaction to other foreign substances. Even monoclonal antibodies against the subject antigen will differ from clone to clone because antibodies produced by different clones
10 may react with different antigenic determinants of the same molecule. From each clone, therefore, it is necessary to obtain the resulting antibody or the antibody-containing medium, serum or ascitic fluid and test its reactivity with the subject biological material and to test its specificity by determining what other biological material, if any, it recognises.

15
When prepared by recombinant DNA techniques, the antibody may be produced by cloning a DNA sequence coding for the antibody or a fragment thereof into a suitable cell, e.g. a microbial, plant, animal or human cell, and culturing the cell under conditions conducive to the production of the antibody or fragment in question and recovering the antibody or
20 fragment thereof from the culture. Possible strategies for the preparation of cloned antibodies are discussed in, for instance, L. Riechmann et al., Nature 332, 24 March 1988, p. 323 ff., describing the preparation of chimeric antibodies of rat variable regions and human constant regions; M. Better et al., Science 240, 20 May 1988, p. 1041 ff., describing the preparation of chimeric mouse-human Fab fragments; A. Sharra and A. Plückthun, Science 240, 20 May
25 1988, pp. 1038-1040, describing the cloning of an immunoglobulin Fv fragment containing antigen-binding variable domains; and E.S. Ward et al., Nature 341, 12 October 1989, pp. 544-546, describing the cloning of isolated antigen-binding variable domains ("single-domain antibodies"). (Humanized monoclonal antibodies in general see, for example, Molecular Biology and Biotechnology (3rd ed.), Walker and Gingold (ed.s), The Royal Society of
30 Chemistry 1993, p 357-385).

Monoclonal antibodies or other genetically engineered antibodies with specificity for the CCF could be used as therapeutic agents. The antibodies should then be humanised to reduce the immunogenicity.

Humanisation is done by grafting the Complementary-Determining Region (CDR) from the original murine antibody to the constant regions of a human antibody. Various methods can be used to ensure the specificity and avidity of the grafted antibody

5 (Queen, C et al, Proc. Natl. Acad. Sci. U.S.A., 86, 10029, 1989 & Reichmann, L. et al, Nature, 332, 323, 1988).

Antibodies to a CCF or preCCF peptide may be used for isolation, for affinity purification, for diagnostic assays, for determination of circulating levels of cancer

10 cachectic factor peptides, and as antagonists to block CCF receptor activity *in vitro* and *in vivo*. (See, for example, Immobilized Affinity Ligand Techniques, Hermanson et al., eds., Academic Press, San Diego, CA, 1992, pp.195-202).

Pharmaceutical compositions comprising CCF peptides

15 In the pharmaceutical composition of the invention, the CCF peptides may be formulated by any of the established methods of formulating pharmaceutical compositions, e.g. as described in Remington's Pharmaceutical Sciences, 1985. The composition may be in a form suited for systemic injection or infusion and may, as such, be formulated with a suitable liquid vehicle such as sterile water or an isotonic saline or glucose solution. The

20 compositions may be sterilized by conventional sterilization techniques which are well known in the art. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with the sterile aqueous solution prior to administration. The composition may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological

25 conditions, such as buffering agents, tonicity adjusting agents and the like, for instance sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc.

The pharmaceutical composition of the present invention may also be adapted for nasal,

30 transdermal, pulmonal or rectal administration. The pharmaceutically acceptable carrier or diluent employed in the composition may be any conventional solid carrier. Examples of solid carriers are lactose, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate and stearic acid. Similarly, the carrier or diluent may include any sustained release material known in the art, such as glyceryl monostearate or glyceryl

35 distearate, alone or mixed with a wax.

It may be of particular advantage to provide the composition of the invention in the form of a sustained release formulation. As such, the composition may be formulated as microcapsules or microparticles containing the peptide encapsulated by or dispersed in a suitable pharmaceutically acceptable biodegradable polymer such as polylactic acid, polyglycolic acid or a lactic acid/glycolic acid copolymer.

For nasal administration, the preparation may contain the peptide dissolved or suspended in a liquid carrier, in particular an aqueous carrier, for aerosol application. The carrier may contain additives such as solubilizing agents, e.g. propylene glycol, surfactants, absorption enhancers such as lecithin (phosphatidylcholine) or cyclodextrin, or preservatives such as parabenes.

Generally, the compounds of the present invention are dispensed in unit dosage form comprising 0.5 μ g -50 mg of the peptide (70 kg patient) together with a pharmaceutically acceptable carrier per unit dosage.

The peptide is considered to be advantageous to use in weight reduction programs, such as for the treatment of diseases or disorders associated with excess, preferably morbid overweight. Examples of such diseases or disorders are obesity and type II diabetes. The dosage of the peptide administered to a patient will vary with the type and severity of the condition to be treated, but is generally in the range of from about 0.01 μ g/kg to about 5 mg/kg body weight.

The peptide is further considered to be advantageous in order to prevent or cure conditions or disorders arising from obesity, and Syndrome X (i.e. obesity, hypertension, insulin resistance, dyslipidermia). Syndrome X is also known as metabolic syndrome or insulin resistance syndrome.

The peptide may be administered in combination with food.

In the pharmaceutical composition of the invention, the peptide may be combined with an appetite-suppressing or satiety-inducing agent. An example of such an agent is GLP-1 which has been shown to have some effect on appetite suppression (cf. M.D. Turton et al., *Nature* 379, 4 January 1996, pp. 69-72).

Agonists and antagonists of CCF peptide

It is further contemplated that the CCF or preCCF peptide in suitably labelled form, e.g. radiolabelled, may be used to identify a receptor for CCF peptide in binding studies using
5 tissue(s) expected to express the receptor, e.g. fat and muscle tissue. Once localized by CCF peptide binding, the receptor may be cloned by expression cloning, i.e. by preparing a cDNA library of the tissue in question, cloning the cDNA into suitable vectors and introducing the vectors into an appropriate cell to effect expression of the cDNA, after which a clone expressing the receptor is identified by binding to CCF
10 peptide. A cell line stably expressing the receptor may then be used in a screening assay for identification of CCF peptide agonists (i.e. compounds acting on the receptor to induce lipolysis or proteolysis, e.g. for the use of weight reduction) or CCF peptide antagonists (i.e. compounds which antagonize the action of CCF peptide on the receptor, e.g. for use in the treatment of cancer cachexia). The terms "antagonist" and "agonist"
15 are used throughout this application to indicate any peptide or non-peptidyl compound which decreases or increases, respectively, the activity of the receptor.

Animal model systems which elucidate the physiological and behavioural roles of the CCF or CCF receptor may be produced by creating transgenic animals in which the
20 activity of the CCF or CCF receptor is either increased or decreased, or the amino acid sequence of the expressed CCF peptide or CCF receptor is altered, by a variety of techniques (see, for example, Molecular Biology and Biotechnology (3rd ed.), Walker and Gingold (ed.s), The Royal Society of Chemistry 1993). Examples of these techniques include, but are not limited to: 1) Insertion of normal or mutant versions of DNA
25 encoding a CCF peptide or CCF receptor, by microinjection, electroporation, retroviral transfection or other means well known to those skilled in the art, into appropriate fertilized embryos in order to produce a transgenic animal; or 2) Homologous recombination of mutant or normal, human or animal versions of these genes with the native gene locus in transgenic animals to alter the regulation of expression or the
30 structure of these CCF peptide or CCF receptor sequences. The technique of homologous recombination is well known in the art. It replaces the native gene with the inserted gene and so is useful for producing an animal that cannot express native CCF peptide or CCF receptors but does express, for example, an inserted mutant CCF receptor, which has replaced the native CCF receptor in the animal's genome by recombination, resulting in
35 underexpression of the transporter. Microinjection adds genes to the genome, but does

not remove them, and so is useful for producing an animal which expresses its own and added CCF peptide or CCF receptors, resulting in overexpression of the CCF peptide or CCF receptors. (For a general description of Embryo Stem Technology see, for example, Smith et al., *Dev. Biol.*, 1992, 151, p 339 and Nichols et al., *Development*, 1990, 110,
5 p 1341).

One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as M2 medium. DNA
10 or cDNA encoding a CCF peptide or CCF receptor is purified from a vector by methods well known in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the transgene. Alternatively or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the trans-gene. The DNA, in an
15 appropriate buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a pipet puller) and the egg to be injected is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (a mouse stimulated by the appropriate hormones to maintain
20 pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develop to term. As noted above, microinjection is not the only method for inserting DNA into the egg cell, and is used/described here only for exemplary purposes. (In general see, for example, Hogan et al., "manipulating the Mouse Embryo - A Laboratory Manual", Cold Spring Harbor Laboratories Press, 1986.)

25 Determining whether a ligand is capable of specifically binding to a CCF receptor may be done by contacting a cell transfected with and expressing DNA encoding the CCF receptor with the ligand under conditions permitting binding of ligands to such receptor, detecting the presence of any such ligand specifically bound to the receptor, and thereby
30 determining whether the ligand specifically binds to the receptor. Alternatively, a cell extract may be prepared from such cells, a membrane fraction from the cell extract may be isolated, and the membrane fraction may be contacted with the ligand under conditions permitting binding of ligands to such receptor, detecting the presence of any such ligand specifically bound to the receptor, and thereby determining whether the ligand
35 specifically binds to the receptor.

Determining whether a ligand is a CCF receptor agonist may be done by contacting a cell transfected with and expressing DNA encoding a CCF receptor with the ligand under conditions permitting activation of said receptor, detecting an increase in activity of said
5 receptor, and thereby determining whether the ligand is a CCF receptor agonist.

Determining whether a ligand is a CCF receptor antagonist can be done by contacting a cell transfected with and expressing DNA encoding a CCF receptor with the ligand in the presence of a known CCF receptor agonist, such as CCF itself, under conditions permitting activation of said receptor, detecting an decrease in activity of said receptor,
10 and thereby determining whether the ligand is a CCF receptor antagonist.

Screening a plurality of chemical compounds or drugs not known to bind to a CCF receptor to identify a compound, or drug, which specifically binds to said receptor, can be done by a) contacting a cell transfected with and expressing CCF receptor with a
15 compound known to bind specifically to the CCF receptor; b) contacting the preparation of step a) with the plurality of compounds, or drugs, not known to bind specifically to the CCF receptor under conditions permitting binding of compounds known to bind the receptor; c) determining whether the binding of the compound, or drug, known to bind to the receptor is reduced in the presence of the compounds, or drugs, relative to the
20 binding of the compound in the absence of the plurality of compounds, or drugs; and if so d) separately determining the binding to the CCF receptor of each compound, or drug, included in the plurality of compounds, or drugs, so as to thereby identify the compound, or drug, which specifically binds to the receptor. Alternatively, a cell extract may be prepared from such transfected cells, a membrane fraction from the cell extract may be
25 isolated, and the membrane fraction may be contacted with the plurality of compounds, or drugs, not known to bind specifically to the CCF receptor under conditions permitting binding of compounds known to bind the receptor, and taken through steps a) to d).

Likewise, screening a plurality of compounds, or drugs, in order to determine agonists to
30 the CCF receptor may be done by a) contacting a cell transfected with and expressing CCF receptor with the plurality of compounds, or drugs, not known to bind specifically to the CCF receptor under conditions permitting activation of the CCF receptor; b) determining whether the activity of the CCF receptor is increased in the presence of the compounds, or drugs; and if so c) separately determining whether the activation of the
35 CCF receptor is increased by each compound, or drug, included in the plurality of

compounds, or drugs, so as to thereby identify the compound, or drug, which activates the receptor.

Alternatively, a cell extract may be prepared from such transfected cells, a membrane fraction from the cell extract may be isolated, and the membrane fraction may be
5 contacted with the plurality of compounds, or drugs, not known to bind specifically to the CCF receptor under conditions permitting activation of the CCF receptor, and taken through steps a) to c).

Screening a plurality of chemical compounds, or drugs, in order to determine antagonists
10 against the CCF receptor may be done by a) contacting a cell transfected with and expressing CCF receptor with the plurality of compounds, or drugs, in the presence of a known CCF receptor agonist, under conditions permitting activation of the CCF receptor; b) determining whether the activity of the CCF receptor is reduced in the presence of the plurality of compounds, or drugs, relative to the activation of the CCF receptor in the
15 absence of the plurality of compounds, or drugs; and if so c) separately determining the inhibition of activation of the CCF receptor for each compound, or drug, included in the plurality of compounds, or drugs, so as to thereby identify the compound, or drug, which inhibits the activation of the receptor.

Alternatively, a cell extract may be prepared from such transfected cells, a membrane
20 fraction from the cell extract may be isolated, and the membrane fraction may be contacted with the plurality of compounds, or drugs, and taken through steps a) to c).

The cDNA sequence may be used to generate antisense molecules either synthetically *in vitro*, or by construction of suitable expression vectors such as retroviral vectors which
25 after introduction into desired tissues may direct the synthesis of antisense RNA *in vivo*. Synthetic antisense molecules may be either DNA, PNA or RNA, or variants hereof. After introduction of the antisense molecules into the desired cells these molecules will act to prevent the production of peptide from the endogenous gene, and thus provide another way to treat cancer cachexia.

30

The term "PNA" (or peptide nucleic acid), is used to indicate a synthetic DNA-mimetic comprising a polyamide backbone bearing ligands at respective spaced locations along the backbone, the ligands being naturally occurring nucleobases, non-naturally occurring nucleobases, or nucleobase-analogues (see, for example, WO92/20703 (Buchard et al.);
35 WO96/02558; WO96/11205).

The term "antisense molecule" is well known to persons skilled in the art. See in general, for example, Molecular Biology and Biotechnology (3rd ed.), Walker and Gingold (ed.s), The Royal Society of Chemistry 1993 or Crooke & Bennett (1996)

- 5 Ann.Rev.Pharmacol.Toxicol. 36, p. 107-129.

An antisense molecule is capable of specifically hybridizing to a unique sequence included within the sequence of a nucleic acid encoding a cancer cachetic factor. As used herein, the phrase "specifically hybridizing" means the ability of a nucleic acid (or peptide nucleic acid) to recognise a nucleic acid sequence complementary to its own and
10 to form double-helical (or triple-helical) segments through hydrogen bonding between complementary base pairs.

The cDNA sequence can also be used to identify the tissues or cells which express the CCF most abundantly. Briefly, mRNA is isolated from various tissues and subjected to
15 PCR analysis or Northern analysis with primers or probes generated from the DNA sequence shown in Fig.No.1. Knowledge of the sites of synthesis may prove very useful for clarifying the function of CCF in health and disease. More specifically, this knowledge is important for designing suitable antisense therapeutic strategies.

- 20 The cDNA sequence may also be used to identify the gene encoding it, as well as its chromosomal location. Apart from providing another means of generating the information necessary for the expression of the peptide as described above, knowledge of the gene and its chromosomal location may be useful in clarifying the mechanisms involved in the generation of cancer cachexia. For example, knowledge of the promoter for this gene
25 may allow the characterisation of specific transcription factors involved in the expression of this gene. In particular, the promoter may be used to screen for inhibitors or activators of transcription. Briefly, the promoter may be linked to a reporter gene such as chloramphenicol acetyl transferase (CAT) or GFP in a suitable vector (see, for example, Lui et al., J.Biol.Chem., 1992, 267, p.11673 ff). When introduced into a suitable cell
30 line, e.g. CHO or other mammalian cell lines, the promoter will give rise to expression of the reporter gene. Natural or synthetic substances may be added to cultures of the expression cell line and their effect on the specific expression from the promoter of the preCCF gene assessed from the level of the reporter molecule. Specific inhibitors may be used to prevent the overexpression of CCF seen in some tumour-bearing patients and thus
35 be a means to treat cancer cachexia. Specific activators may be used to induce

overexpression in morbidly obese patients and thus be a means to promote weight loss.

The invention is further illustrated in the following examples which are not in any way intended to limit the scope of the invention as claimed.

5

EXAMPLES

Example 1.

10 Isolation and characterization of human cDNA clones encoding a peptide precursor with homology to a murine CCF.

The published 20 aa sequence, Tyr-Asp-Pro-Glu-Ala-Ala-Ser-Ala-Pro-Gly-Ser-Gly-Asp-Pro-Ser-His-Glu-Ala-(Ser)-(Ala), for the core peptide of the murine 24kD cancer
15 cachexia factor (Todorov et al, op.cit) was used as a search string to search DNA sequence databases for matching sequences, using a search program that translates the DNA sequences of the database into encoded amino acid sequence. One almost perfect matching sequence was found in an expressed sequence tag (EST) database representing cDNA from breast tumour tissue of a 55 year old caucasian woman. There were two
20 mismatches between the translated sequence of the EST and the published cancer cachexia factor 20aa sequence: Asp-13 of the published sequence was Asn in the EST, and Ser-15 was Cys in the EST.

The matching cDNA clone was named p24k-inc (pND-N6, INC607227). The complete
25 sequence of the cDNA insert in the p24k-inc plasmid was determined, using standard sequencing primers starting at either side of the insert in the vector (pSPORT1, Gibco-BRL), a Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham, UK) and an ALF DNA sequencer (Pharmacia, Sweden). The DNA sequence shown in SEQ ID NO:1 was obtained. The nucleotides encoding the two divergent amino acids
30 were identical to those found in the original EST sequence, indicating that the divergence was not due to DNA sequencing errors. The cDNA sequence specified a 126aa open reading frame containing the search sequence, shown in bold in SEQ ID NO:1 and with the two divergent amino acids underlined. As indicated in SEQ ID NO:1, there were stop codons downstream from the search sequence, but there were no stop codons in the open
35 frame upstream from the search sequence, thus the open frame could potentially be the

3'-end of a larger coding region stretching further towards a 5'-end.

In order to characterize the 5'-end of the CCF-related open reading frame, sets of PCR primers were designed from the obtained cDNA sequence and used to generate new and longer cDNA clones. One set of primers, designated 24k-B2 (SEQ ID NO:6) and 24K-B4 (SEQ ID NO:7), was designed to be used in a 5' RACE (rapid amplification of cDNA ends) together with the primers AP1 (SEQ ID NO:8) and AP2 (SEQ ID NO:9) provided with marathon-ready cDNA from Clontech Laboratories Inc. (Palo Alto, CA). Marathon-ready cDNA from human testis (Clontech) was used as template. The PCR reaction was carried out according to the instruction manual provided with the marathon-ready cDNA. In the first reaction 5 ml marathon-ready cDNA was used with the primer set 24k-B2 and AP1. The reaction mixture was incubated in a Hybaid Touchdown PCR machine (AH Diagnostics, Denmark) for 1 minute at 94°C, then for 5 cycles of 94°C, 30 seconds, 72°C 4 minutes, 5 cycles of 94°C, 30 seconds, 70°C 4 minutes, 25 cycles of 94°C, 30 seconds, 68°C 4 minutes. An aliquot of the reaction mixture was analyzed by electrophoresis on a 1% agarose gel and a faint smear was observed. 2 ml of a 50 fold dilution of the first reaction mixture was used for a nested primer reaction with the primer set 24K-B4 and AP2, using the same incubation conditions as before. Analysis of the reaction mixture by electrophoresis on a 2% agarose gel showed a discrete band of about 400 bp. The band was cut out of the gel, and the DNA was purified using JETsorb DNA extraction kit (Genomed, Germany). The purified DNA was cloned using a TA Cloning™ Kit with vector pCR2.1 and *E.coli* OneShot™ cells (Invitrogen, CA). Individual white colonies representing recombinants were isolated, and plasmid DNA was prepared using the Wizard™ plasmid purification kit (Promega, WI). 10 individual plasmids were subjected to DNA sequencing using standard sequencing primers starting at either side of the insert in the vector, as described above. Two types of sequences with extensive homology to each other were obtained. An example of each sequence is shown in SEQ ID NO:3 and SEQ ID NO:4, respectively. The sequences were identical over most of their lengths and only diverged in the penultimate 5'-end. Examination of the sequences with respect to the open reading frame containing the original 20 aa search sequence described above, showed that both types contained a stop codon in this frame four codons upstream from the first codon known from clone p24k-inc. Thus the maximal extent of the CCF open reading frame has been established. When counting from the first potential initiator Methionine in this frame (shown in *italics* in SEQ ID NO:1) the CCF-related cDNA can be seen to encode a 110 aa long peptide shown in SEQ ID NO:2, again with the search

sequence shown in bold. The 110 aa peptide (named preCCF) has the characteristics of a secretory peptide, with amino acid residues 1-18 constituting a potential signal sequence.

In order to generate independent clones containing the complete preCCF coding region
5 from testis-derived cDNA, a set of primers, designated 24k-F (SEQ ID NO:10) and 24K-
B7 (SEQ ID NO:11), was designed. The template for this reaction was single stranded
cDNA prepared from 1 mg testis polyA+ RNA by oligo-dT priming using 1st-strand™
cDNA Synthesis Kit (Clontech). The PCR reaction was carried out with Pfu Polymerase
(Stratagene, Germany) according to the protocol supplied by the manufacturer. The
10 reaction mixture was incubated in a Hybaid Touchdown PCR machine (AH Diagnostics,
Denmark) for 5 minute at 94°C, then for 30 cycles of 94°C, 30 seconds, 66°C 30
seconds, 75°C 45 seconds. The reaction mixture was analyzed by electrophoresis on a 2%
agarose gel. A band of the expected size (400 bp) was seen. As preparation for cloning in
the TA™ Cloning Vector kit, the PCR reaction mixture was treated with AmpliTaq
15 polymerase for 8 minutes at 72°C. The DNA was immediately purified by phenol
extraction and ethanol precipitation and cloned into the pCR2.1 vector as described for
the 5' RACE product. Individual white colonies representing recombinants were isolated,
and plasmid DNA was prepared and sequenced as described for the cloned 5' RACE
products. The inserts of 5 individual clones were sequenced; all gave the same sequence,
20 shown in SEQ ID NO:5. The sequence obtained was 100% identical to the corresponding
sequence from p24k-inc.

Example 2.

25 Expression of recombinant preCCF in mammalian cells.

- A DNA fragment coding for preCCF flanked with the restriction site HindIII in its 5'-end and an EcoRI site in its 3'-end was generated by PCR. The template for the PCR reaction was plasmid p24k-inc linearized by digestion with the restriction enzyme NotI, and the primers were 24k-Fex1 (SEQ ID NO:12) and 24k-Bex1 (SEQ ID 13). The PCR reaction
- 5 was carried out with Pfu Polymerase (Stratagene, Germany) according to the protocol supplied by the manufacturer. The reaction mixture was incubated in a Hybaid Touchdown PCR machine (AH Diagnostics, Denmark) for 5 minute at 94°C, then for 30 cycles of 94°C, 30 seconds, 56°C 45 seconds, 75°C 45 seconds and finally for 5 minutes at 72°C. The reaction mixture was analyzed by electrophoresis on a 2% agarose gel. A
- 10 band of the expected size (366 bp) was seen. The reaction mixture was treated with the restriction enzymes HindIII and EcoRI to give a 355 bp fragment, as recommended by the supplier (New England Biolab, Ma). The resulting digestion products were electrophoresed on a 2% agarose gel, and the 355 bp band was cut out and purified using JETsorb DNA extraction kit (Genomed, Germany). The fragment was ligated into a
- 15 HindIII - EcoRI cut pcDNA3 vector (Invitrogen, CA) and transformed into *E.coli* DH5a cells. pcDNA3 is a mammalian expression vector, using the human CMV promoter to drive expression, and further containing the neomycin gene to allow for selection of the plasmid in mammalian cells. Individual transformant clones were isolated and plasmid DNA was prepared using the Wizard™ plasmid purification kit (Promega, WI). One
- 20 preparation, which upon restriction enzyme digestion gave the expected fragments, was subjected to DNA sequencing of the preCCF coding region to confirm that no mutations had occurred during the construction of the plasmid. The selected plasmid was named pcDNA-24k1.
- 25 pcDNA-24k1 was used to transform the Chinese Hamster Ovary cell line DG44 and the human liver cell line HEK293. Cells were selected and propagated in standard growth medium containing fetal calf serum in the presence of 700 µg/ml Geneticin (Gibco). Individual cells were isolated and expanded to clonal cell lines expressing preCCF. PreCCFa expressing clones showed slightly altered growth characteristics compared with
- 30 vector-transfected cells, indicating preCCF mediated perturbation of normal growth.

One clone was propagated in serum-free medium. The medium was collected, dialyzed against water and concentrated 100 fold. An aliquot of the concentrate was subjected to Western analysis (Current Protocols in Molecular Biology on CD-ROM, eds. Ausubel at

35 al., 1996, unit 10.8) using an antibody raised against a synthetic peptide corresponding to

SEQ ID NO:18 (see example 7). A band of 17kD, which was not present in media from control cells, was detected.

5 Example 3.

Expression of recombinant preCCF in insect cells.

pcDNA-24k1 was digested with HindIII and incubated with Klenow DNA polymerase in the presence of all four dNTPs to generate a blunt-end (5') next to the initiator codon for
10 preCCF. The resulting linearized plasmid DNA was purified by phenol extraction and ethanol precipitation, and then digested with the restriction enzyme EcoRI. The digestion mixture was electrophoresed on a 2% agarose gel, and the 350 bp band was cut out and purified using JETsorb DNA extraction kit (Genomed, Germany). The fragment was cloned into SmaI - EcoRI cut pVL1393 vector (Invitrogen, CA) and *E.coli* DH5a cells.
15 pVL1393 is a Baculovirus transfer expression vector, using the Baculovirus Polyhedron protein promoter to drive expression; further, it contains a Baculovirus-derived DNA sequence which allows integration of the transfer vector into the wt Baculovirus genome. Individual transformants of *E.coli* DH5a were isolated and plasmid DNA was prepared using the Wizard™ plasmid purification kit (Promega, WI). One preparation which upon
20 restriction enzyme digestion gave the expected fragments was named pVL-24k1.

pVL-24k1 was used to transform the SF9 insect cell line together with linear AcMNPV DNA (Invitrogen). Culture supernatant from transfected SF9 cells was collected after 5 days, and was used in various dilutions to infect fresh monolayers of SF9 cells plated in
25 100 mm dishes. The infected cells were overlaid with 1.5 % agarose containing complete TNM-FH medium. After 6 days, nine presumed recombinant plaques were identified by their occlusion-negative phenotype and used to infect 6-well plates containing SF9 cells. After 5 days, the corresponding virus DNA was purified and subjected to a PCR reaction with forward and reverse primers flanking the site of recombination in the virus DNA.
30 After loading of the PCR products on agarose gel, it was seen that all nine virus-isolates were recombinants. In order to evaluate expressed preCCF, SF9 cells seeded in 6-well plates were infected with the nine virus-isolates (and with virus expressing beta-galactosidase as control). 40 hours post-infection, the wells were incubated in 1 ml of complete TNM-FH medium containing 40 µCi ³⁵S-Met and 40 µCi ³⁵S-Cys (Amersham).
35 After 5 hours, the cells were loosened with cell-scrapers and 100 µl cell suspensions were

transferred to Eppendorph tubes and centrifuged at 6000 rpm. To the cell pellets were added 2xSDS sample buffer with 100 mM DTT and the tubes were placed in boiling water for three minutes before samples were loaded onto a 15 % SDS-PAGE gel. Before drying the gel, it was incubated in 1 M salicylic acid for 30 minutes. The gel was
5 exposed to X-ray film at -80°C for 24 hours. As seen in Fig. 4, all virus-isolates (minus isolate #2) expresses preCCF with a molecular weight of approx. 17 kD, corresponding to the band which was seen in Western analysis of material from mammalian cells.

10 Example 4.

Analysis of preCCF related mRNA expression in normal and cancerous tissues and cell lines.

Reverse transcriptase (Rt) PCR and Northern analysis has been used to examine the
15 expression of preCCF related mRNA expression in a wide range of tissues and cell lines.

For RtPCR analysis 50 ng to 1 µg purified polyA+ RNA or total RNA was converted to single stranded cDNA by oligo-dT priming using 1st-strand™ cDNA Synthesis Kit (Clontech), whereupon two primers, 24k-F (SEQ ID NO:10) and 24k-B1 (SEQ ID
20 NO:14) were used in a PCR reaction which typically consisted of 1 cycle of 94°C for 5', 30 cycles of 94°C for 30'', 66°C for 45'' and 72°C for 45'', and one final cycle of 72°C for 5'. A DNA fragment of 156 bp was expected to be amplified, if the preCCF mRNA had been present in sufficient amounts in the original sample. A high amount of a 156 bp fragment was obtained in reactions where 1 µg testis polyA+ RNA was used to generate
25 the input cDNA. Lower amounts or no fragment were seen from testis cDNA if less polyA+ RNA or total RNA had been used to generate the input cDNA. A low amount of fragment was also detected from skeletal muscle-derived cDNA. None of the other tested cDNA preparations gave rise to the 156 bp fragment. Tested cDNA from non-tumorous tissues were derived from commercially available RNA preparations (Clontech) from
30 human placenta, testis, ovary, pancreas, skeletal muscle, liver, lung, spleen, kidney, heart, brain and colon. (It should be noted that in most cases total RNA was used for the analysis, ie. preCCF mRNA may have been present at too low a concentration to allow detection). In addition to these normal tissues, several human tumours were also tested, including an insulinoma, a glucagonoma, and several different melanomas, colon and
35 lung tumours. None of these gave rise to the 156 bp fragment (not even when 1 µg

polyA+ RNA was used as template to generate the cDNA, eg. for insulinoma, glucagonoma and colon tumors). It is concluded that none of the analyzed tissues synthesize abundant amounts of the preCCF mRNA. We have not been able to ascertain whether or not the analyzed tumours gave rise to cachexia *in vivo*.

5

For Northern analysis, commercially available blots (Clontech) were hybridized to a SalI - ScaI 390 bp fragment containing the preCCF coding region from p24k-inc. The 390 bp DNA fragment was purified by gel electrophoreses followed by JETsorb as described previously, and labelled with alpha-³²P-dATP by random priming. The blots were
10 prehybridized in 0.5 M NaH₂PO₄, pH 7.4, 1 mM EDTA, 7% SDS for 1-2 hours at 65°C, then hybridized in the same mixture containing a probe concentration of ca. 1 x 10⁷ cpm/ml overnight. After hybridization the blots were rinsed in 2xSSC at room temperature, then washed 2x10 minutes in the prehybridization mixture at 65°C. The blots were exposed to PhosphorImager screens (Molecular Dynamics) or X-ray films
15 (Fuji).

The blots used contained in each lane 2 µg polyA+ RNA from the following tissues:

- MTN 1 (Cat #7760-1): heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas
- MTN 2 (Cat #7759-1): Spleen, thymus, prostate, testis, ovary, small intestine, colon,
20 peripheral blood leukocytes
- Cancer cell lines (Cat # 7757-1): promyelocytic leukemia HL-60, HeLa cell S3, chronic myelogenous leukemia K-562, lymphoblastic leukemia MOLT-4, Burkitt's lymphoma Raji, colorectal adenocarcinoma SW480, lung carcinoma A549, melanoma G361.
- Endocrine tissues (Cat # 7751): pancreas, adrenal medulla, thyroid, adrenal cortex,
25 testis, thymus, small intestine, stomach
- Muscle tissues (Cat # 7765): Skeletal muscle, uterus, colon, small intestine, bladder, heart, stomach, prostate.

Upon long exposure of the hybridized blots, a band of 1.7kb was seen in the two cancer cell lines: lymphoblastic leukemia MOLT-4 and Burkitt's lymphoma Raji. A similar, but
30 much fainter band was also observed in colorectal adenocarcinoma SW480 & lung carcinoma A549. A faint band of about 0.5 kb was seen in several samples (kidney, adrenal medulla and cortex, prostate, heart and colon). This band was in all case located at the end of the gel, and may be either an artefact when present or have been cut off on the other lanes. No band was seen in testis, although the PCR analysis indicated the
35 presence of a preCCF-related mRNA of at least 700 bp in this tissue. PCR analysis is

known to be more sensitive than Northern analysis, which may account for the difference. Alternatively, the difference may be explained by the fact that different mRNA preparations were used for the PCR and the Northern analysis.

- 5 In conclusion, the PCR and the Northern analysis indicate that preCCF mRNA is expressed at undetectable to very low levels in most normal and cancerous tissues. However, a few cancer cell lines expressed the mRNA at more abundant levels. This observation supports the notion that abundant expression of the 24k mRNA is related to certain types or stages of tumor development.

10

Example 5.

Determination of chromosomal location and structure of the human preCCF gene.

- 15 The chromosomal location of the gene encoding preCCF was determined using PCR analysis. Genomic DNA isolated from hybrid mouse cell lines each containing one human chromosome (Cell Hybrid Mapping Panel #2, NIGMS Human Genetics Mutant Cell Repository, NJ) was used as template for a PCR reaction using primers 24k-F and 24k-B1 (SEQ ID NO:14). The PCR reaction was performed using Taq polymerase and a
20 reaction profile of 95°C for 3 minutes, 30 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 4 minutes, followed by 72°C for 10 minutes. This primer set generates a 156 bp fragment from cloned preCCF cDNA, whereas it was shown to generate a 4 kb fragment on human genomic DNA, indicating the presence of at least one intron in the gene encoding the cDNA. Only DNA from the mouse-human hybrid cell line containing
25 human chromosome no.10 gave the 4 kb fragment, indicating that the gene is located on this chromosome.

- Southern analysis of human DNA cut with various restriction enzymes (Clontech human genoblot, Cat # 7700-1, 4 mg digested Human DNA/lane) using the 390 bp DNA probe
30 fragment and the hybridization conditions described above showed that only specific and distinct bands hybridized to the probe under stringent conditions, indicating that the gene is a single copy gene and not closely related to a gene family. The following genomic fragments were shown to hybridize:

- 35 EcoRI: 5.5 + 1.7 kb; HindIII: 4.4 + 3.5 kb; BamHI: 9 kb; PstI: 3.8 kb; BglII: 1.9 +

ca. 1.4 kb.

5 Example 6.

Identification of homologous genes from other species.

Southern analysis of EcoRI digested genomic DNA from several species (Clontech Zoo-
blot, Cat # 7753-1, human, monkey, rat, mouse, dog, cow, rabbit, chicken, yeast) using
10 the 390 bp DNA probe fragment and the hybridization conditions described in Example 5
showed strong hybridization to a 5.5 kb and a 1.7 kb fragment from human DNA and to
a 7.7 kb and a 5.5 kb fragment from monkey DNA, and fainter hybridization to a 3 kb
fragment from cow and a 6.7 kb fragment from rabbit. None of the other species showed
any specific hybridization under stringent conditions. This indicates that a homologous
15 gene is present in several other mammalian species

Example 7.

Production of antibodies against preCCF peptides.

20 One peptide with the sequence YDPEAASAPGSGNPCHEASA (which is located near the
N-terminus of preCCF) and one peptide with the sequence
LESVGKGAVHDVKDVLDSVL (which is located at the C-terminus of preCCF) were
made synthetically (Quality Controlled Biochemicals Inc., MA). The peptides were
coupled to the carrier molecule Ovalbumine by carbodiimide (EDC). For each peptide,
25 two NZW rabbits were immunized subcutaneously with 100 mg carrier-coupled peptide in
Freunds Complete Adjuvant and boosted with the same mixture with two weeks intervals.
The rabbits were bled 10 days after the last immunization, and serum was stored at -
20°C.

101 DVKDVLDVSVL

MRFMILLFLT ALAGALVC: potential signal sequence

K/R: potential basic protease cleavage site

*: potential O-glycosylation site

#: potential N-glycosylation site

5

SEQ ID NO: 3

Testis 5' RACE clone type 1.

i) Sequence characteristics:

length: 362 base pairs

type: nucleic acid

strandedness: double

topology: linear

10

ii) Molecule type: cDNA

```

CTGGGAGGCGGCTCCCTCAAGCACTTTAAACCTCATTACGCAGCGGCAGCCTCCACAT
1  -----+-----+-----+-----+-----+-----+-----+-----+ 60
W E A A P L K H F K P H S R S G S L H I -
ATTGAGAACGGATCTTGGGACTGGGGAGAAGCAGAAAACCTACAAGGCTGGAATAATTGT
15 61  -----+-----+-----+-----+-----+-----+-----+-----+ 120
L R T D L G T G E K Q K T Y K A G K I V -
GGCTCTGAGGTGGGAGAAGAGGAGGAGGAGAACACACCACTGGAAGACTGGTTCCCATTTG
121 -----+-----+-----+-----+-----+-----+-----+-----+ 180
A L R W E K R R R R T H H W K T G S H W -
GTCCTGTCTATGCTTAAAAAAGGCCAGAGAGGCAGTCTTGACACCTTAGATCCCAAGA
181 -----+-----+-----+-----+-----+-----+-----+-----+ 240
S L S C L K K G P E R Q S * H P R S Q D -
TCTCCAAGGATTTGGTGGCATACCACTCCAGCACACAGAAGCATGAGGTTTCATGACTCT
241 -----+-----+-----+-----+-----+-----+-----+-----+ 300
L Q G F G G I P T P A H R S M R F M T L -
CCTCTTCTGACAGCTCTGGCAGGAGCCCTGGTCTGTGCCTATGATCCAGAGGCCGCCTC
301 -----+-----+-----+-----+-----+-----+-----+-----+ 360
L F L T A L A G A L V C A Y D P E A A S -
TG
361 -- 362

```

25

SEQ ID NO: 4

Testis 5' RACE clone type 2.

i) Sequence characteristics:

length: 332 base pairs

type: nucleic acid

strandedness: double

topology: linear

30

ii) Molecule type: cDNA

```

GGGTGAGGAGAGAGGAGGGTGAAGACCGTCAGTCCAGGGCATGTACTCAGAACGCTCTCT
1  -----+-----+-----+-----+-----+-----+-----+-----+ 60
G E E R G G * R P S V Q G M Y S E R S L -
GGAAAGCCAGCCTTTGTTGACTTAACAGGTGGCTCTGAGGTGGGAGAAGAGGAGGAGGAG
61  -----+-----+-----+-----+-----+-----+-----+-----+ 120
E S Q P L L T * Q V A L R W E K R R R R -
AACACACCACTGGAAGACTGGTTCCCATTTGGTCCCTGTCTGCTTAAAAAAGGCCAGAG
121 -----+-----+-----+-----+-----+-----+-----+-----+ 180

```

35

5

Testis-derived preCCP coding region.

15

20

25

PCR primer 24K-B2.

35

5' - CTG ATG CTT CAT GGC AAG GGT TCC CC

SEQ ID NO: 7

PCR primer 24K-B4.

- i) Sequence characteristics:
length: 24 bases
type: nucleic acid
strandedness: single

- ii) Molecule type: synthetic DNA

5'- CAG AGG CGG CCT CTG GAT CAT AGG

SEQ ID NO: 8

PCR primer AP1

- i) Sequence characteristics:
length: 27 bases
type: nucleic acid
strandedness: single

- ii) Molecule type: synthetic DNA

1 CCATCCTAAT ACGACTCACT ATAGGGC

SEQ ID NO: 9

PCR primer AP2

- i) Sequence characteristics:
length: 24 bases
type: nucleic acid
strandedness: single

- ii) Molecule type: synthetic DNA

GACTCACT ATAGGGCTCG AGCGGC

SEQ ID NO: 10

PCR primer 24K-F.

- i) Sequence characteristics:
length: 21 bases
type: nucleic acid
strandedness: single

- ii) Molecule type: synthetic DNA

5'-GAT CTC CAA GGA TTC GGT GGC

SEQ ID NO: 11

PCR primer 24K-B7.

- i) Sequence characteristics:
length: 27 bases
type: nucleic acid
strandedness: single

- ii) Molecule type: synthetic DNA

5'- CTC AGC TTC TCC TTA CAG CTA TAG TAC

SEQ ID NO: 12

PCR primer 24K-Fex.

- i) Sequence characteristics:

5 length: 37 bases
 type: nucleic acid
 strandedness: single

ii) Molecule type: synthetic DNA

5'-CCG CCA CAA GCT TCC ACC ATG AGG TTC ATG ACT CTC C

10 SEQ ID NO: 13
 PCR primer 24K-Bex.

i) Sequence characteristics:
 length: 31 bases
 type: nucleic acid
 strandedness: single

ii) Molecule type: synthetic DNA

15 5'-CGG GAA TTC CTT ACA GCT ATA GTA CTG AGT C

SEQ ID NO: 14
 PCR primer 24K-B1.

20 i) Sequence characteristics:
 length: 21 bases
 type: nucleic acid
 strandedness: single

ii) Molecule type: synthetic DNA

5'-GCT TCA TGG CAA GGG TTC CCC

25

30

35

SEQ ID NO: 15

cDNA of the 24 aa cancer cachetic factor.

- 5 i) Sequence characteristics:
length: 72 base pairs
type: nucleic acid
strandedness: double
topology: linear

- ii) Molecular type: cDNA

10 GCCTATGATCCAGAGGCCGCTCTGCCCCAGGATCGGGGAACCCCTTGCCATGAAGCATCA 60
1 -----
A Y D P E A A S A P G S G N P C H E A S
GCAGCTCAAAAG
61 ----- 72
A A Q K

15 SEQ ID NO: 16

cDNA of the 20 aa cancer cachetic factor.

- 20 i) Sequence characteristics:
length: 60 base pairs
type: nucleic acid
strandedness: double
topology: linear

- ii) Molecular type: cDNA

25 TATGATCCAGAGGCCGCTCTGCCCCAGGATCGGGGAACCCCTTGCCATGAAGCATCAGCA 60
1 -----
Y D P E A A S A P G S G N P C H E A S A

SEQ ID NO: 17

The 24 aa cancer cachetic factor.

- 30 i) Sequence characteristics:
length: 24 aa
type: amino acid
topology: linear

- ii) Molecular type: protein

35 1 AYDPEAASAP GSGNPCHEAS AAQK

SEQ ID NO: 18

The 20 aa cancer cachetic factor.

- i) Sequence characteristics:

length: 20 aa
type: amino acid
topology: linear

5

ii) Molecular type: protein.

1 YDPEAASAPG SGNPCHEASA

10

15

20

25

30

35

CLAIMS

1. A DNA construct comprising a DNA sequence encoding a cancer cachectic factor (CCF).
- 5 2. A DNA construct according to claim 1, wherein the DNA sequence is a chromosomal DNA sequence.
3. A DNA construct according to claim 1, wherein the DNA sequence is a cDNA sequence.
- 10 4. A DNA construct according to claim 3, wherein the DNA sequence is a human cDNA sequence.
5. A DNA construct according to claim 1, wherein the DNA sequence is the DNA sequence shown in SEQ ID NO:15, or a DNA sequence which hybridises to the DNA sequence shown
15 in SEQ ID NO:15, or a DNA sequence which has a sequence homology of at least 60% to the DNA sequence shown in SEQ ID NO:15.
6. A DNA construct according to claim 1, wherein the DNA sequence is the DNA sequence shown in SEQ ID NO:16, or a DNA sequence which hybridises to the DNA sequence shown
20 in SEQ ID NO:16, or a DNA sequence which has a sequence homology of at least 60% to the DNA sequence shown in SEQ ID NO:16.
7. A DNA construct comprising a DNA sequence encoding a precursor of a cancer cachectic factor (preCCF).
- 25 8. A DNA construct according to claim 7, wherein the DNA sequence is a chromosomal DNA sequence.
9. A DNA construct according to claim 7, wherein the DNA sequence is a cDNA sequence.
- 30 10. A DNA construct according to claim 9, wherein the DNA sequence is a human cDNA sequence.
11. A DNA construct according to claim 7, wherein the DNA sequence is the DNA sequence
35 shown in SEQ ID NO:1, or a DNA sequence which hybridises to the DNA sequence shown

in SEQ ID NO:1, or a DNA sequence which has a sequence homology of at least 60% to the DNA sequence shown in SEQ ID NO:1.

12. A recombinant vector comprising a DNA construct of claim 1 or 7.
- 5 13. A recombinant host cell comprising a DNA construct according to claim 1 or 7, or a vector according to claim 12.
14. A host cell according to claim 13 which is a microbial, plant, insect or mammalian cell.
- 10 15. A method of producing a cancer cachectic factor, wherein a cell according to claim 13 is cultured in a suitable culture medium under conditions permitting expression of the DNA coding for the cancer cachectic factor and recovering the resulting cancer cachectic factor from the culture.
- 15 16. A method according to claim 15 including the step of processing the precursor of the cancer cachectic factor (preCCF).
17. An isolated cancer cachectic factor which has the amino acid sequence: Tyr Asp Pro Glu
20 Ala Ala Ser Ala Pro Gly Ser Gly Asn Pro Cys His Glu Ala Ser Ala (SEQ ID NO: 17).
18. An isolated cancer cachectic factor which has the amino acid sequence: Ala Tyr Asp Pro
Glu Ala Ala Ser Ala Pro Gly Ser Gly Asn Pro Cys His Glu Ala Ser Ala Ala Glu Lys (SEQ
ID NO: 18).
- 25 19. Glycosylated forms of the cancer cachectic factor having essentially the same biological activity as the CCF-isolated form from tumor tissue.
20. A cancer cachectic factor according to claim 19 which is O-glycosylated at Ser-7.
- 30 21. A cancer cachectic factor according to claim 19 which is O-glycosylated at Ser-11.
22. A cancer cachectic factor according to claim 19 which is O-glycosylated at Ser-20.
- 35 23. A cancer cachectic factor according to claim 19 which is O-glycosylated at one or more of

residues Ser-7, Ser-11 and Ser-20.

24. A cancer cachectic factor according to claim 19 which is N-glycosylated at residue Asn-13.

5

25. A cancer cachectic factor according to claim 19 which is O-glycosylated at one or more of residues Ser-7, Ser-11 and Ser-20, and N-glycosylated in Asn-13 .

26. An isolated cancer cachectic factor encoded by the DNA sequence of claim 5, 6 or 11.

10

27. An isolated polypeptide which is a precursor of a cancer cachectic factor.

28. A preCCF polypeptide according to claim 27 which is of human origin.

15 29. A preCCF polypeptide according to claim 27 which has the amino acid sequence shown in SEQ ID NO: 2, or which is a natural variant of the polypeptide with the amino acid sequence shown in SEQ ID NO: 2 or which has a sequence homology of at least 60% to the amino acid sequence shown in SEQ ID NO: 2.

20 30. A preCCF polypeptide according to claim 27 encoded by the DNA sequence of claim 9.

31. A pharmaceutical composition comprising a cancer cachectic factor together with a pharmaceutically acceptable diluent or carrier.

25 32. A pharmaceutical composition according to claim 31 comprising an amount of the cancer cachectic factor sufficient to induce weight loss in a subject to whom it is administered.

33. A pharmaceutical composition according to claim 31 additionally comprising a substance capable of reducing appetite or inducing satiety.

30

34. A pharmaceutical composition according to claim 33, wherein the substance capable of reducing appetite or inducing satiety is glucagon-like peptide 1 or glucagon-like peptide 2.

35 35. A pharmaceutical composition according to claim 31 additionally comprising a substance capable of increasing the ratio of lean body mass to fat body mass.

36. A pharmaceutical composition according to claim 35, wherein the substance capable of increasing the ratio of lean body mass to fat body mass is growth hormone or a growth hormone secretagogue.
- 5
37. A method of treating conditions or disorders arising from obesity, the method comprising administration to a subject in need of such treatment of an amount of a cancer cachectic factor sufficient to alleviate said condition.
- 10
38. A method of inducing weight loss in a subject, the method comprising administration to a subject of an amount of a cancer cachectic factor sufficient to induce weight loss in said subject.
- 15
39. A method according to any of claims 37-38 additionally comprising administration of a substance capable of reducing appetite or inducing satiety.
40. A method according to claim 39, wherein the substance capable of reducing appetite or inducing satiety is glucagon-like peptide 1 or glucagon-like peptide 2.
- 20
41. A method according to any of claims 37-38 additionally comprising administration of a substance capable of increasing the ratio of lean body mass to fat body mass.
42. A method according to claim 41, wherein the substance capable of increasing the ratio of lean body mass to fat body mass is growth hormone or a growth hormone secretagogue.
- 25
43. A method of preventing or treating non-insulin dependent diabetes (NIDDM) comprising administration to a subject exhibiting symptoms of NIDDM of an amount of a cancer cachectic factor sufficient to alleviate said symptoms.
- 30
44. A method of preventing or treating Syndrome X comprising administration to a subject exhibiting symptoms of Syndrome X of an amount of a cancer cachectic factor sufficient to alleviate said symptoms.
- 35
45. Use of a cancer cachectic factor to prepare a medicament for the prevention or treatment of conditions or disorders arising from obesity.

46. Use of a cancer cachectic factor to prepare a medicament for inducing weight loss in a subject.
- 5 47. Use of a cancer cachectic factor to prepare a medicament for the prevention or treatment of non-insulin dependent diabetes.
48. Use of a cancer cachectic factor to prepare a medicament for the prevention or treatment of Syndrome X.
- 10 49. An antibody capable of binding to a cancer cachectic factor or a precursor hereof.
50. An antibody according to claim 49, wherein the antibody is a monoclonal antibody
- 15 51. An antibody according to claim 49, for direct application in cancer patients in order to reduce weight loss and tumor growth.
52. A method for preventing unwanted endogenous synthesis or activity of the cancer cachectic factor, e.g. in potential cancer patients, thus preventing weight loss (tissue
20 degeneration) comprising inhibiting transcription of the precursor gene of p24CCF or inhibiting the processing of the precursor protein to yield mature active p24CCF or other steps.
53. A method according to claim 52 comprising administering an antisense molecule
25 capable of specifically hybridizing to the sequence of a nucleic acid encoding the cancer cachectic factor so as to prevent translation of the mRNA molecule to a subject in need thereof.
54. Use of a CCF or preCCF peptide in suitably labelled form, preferably radiolabelled
30 form, to identify a receptor for CCF peptide in binding studies using tissue(s) expected to express the receptor, preferably fat and muscle tissue.
55. A method for detecting a CCF24kD CxP core peptide agonists or preferably a CCF24kD CxP core peptide antagonists comprising contacting a CCF receptor according
35 to claim 54 or a cell transformed with a DNA molecule capable of expressing CCF

receptor with a test agent under conditions enabling the activation of the CCF receptor, and detecting an increase or decrease in CCF receptor activity.

56. Method of diagnosing cancer comprising detecting the presence of p24CCF in urine
5 or serum of potential cancer patients, preferably by ELISA using specific antibodies to p24CCF.

57. Diagnostic tool or test kit for diagnosing cancer comprising specific antibodies to p24CCF.

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SEQ ID NO: 1

cDNA insert of clone p24k-inc with translation in the frame containing the 20aa search string.

i) Sequence characteristics:

length: 515 base pairs
 type: nucleic acid
 strandedness: double
 topology: linear

ii) Molecule type: cDNA

```

CAAGATCTCCAAGGATTCGGTGGCATACCCACTCCAGCACACAGAAGCATGAGGTTTCATG
1  -----+-----+-----+-----+-----+-----+-----+-----+ 60
  Q D L Q G F G G I P T P A E R S M R F M -
ACTCTCCTCTTCCTGACAGCTCTGGCAGGAGCCCTGGTCTGTGCTATGATCCAGAGGCC
61  -----+-----+-----+-----+-----+-----+-----+-----+ 120
  T L L F L T A L A G A L V C A Y D P E A -
GCCTCTGCCCCAGGATCGGGGAACCCCTTGCCATGAAGCATCAGCAGCTCAGAAGGAAAAT
121 -----+-----+-----+-----+-----+-----+-----+-----+ 180
  A S A P G S G N P C H E A S A A Q K E N -
GCAGGTGAAGACCCAGGGTTAGCCAGACAGGCACCAAGCCAAGGAAGCAGAGATCCAGC
181 -----+-----+-----+-----+-----+-----+-----+-----+ 240
  A G E D P G L A R Q A P K P R K Q R S S -
CTTCTGGA AAAAGGCCTAGACGGAGCAAAAAAGCTGTGGGGGGACTCGGAAAAGTAGGA
241 -----+-----+-----+-----+-----+-----+-----+-----+ 300
  L L E K G L D G A K K A V G G L G K L G -
AAAGATGCAGTCGAAGATCTAGAAAGCGTGGGTAAAGGAGCCGTCCATGACGTTAAAGAC
301 -----+-----+-----+-----+-----+-----+-----+-----+ 360
  K D A V E D L E S V G K G A V H D V K D -
GTCCTTGACTCAGTACTATAGCTGTAAGGAGAGGCTGAGAAATGATACCCAGGAGCAGCA
361 -----+-----+-----+-----+-----+-----+-----+-----+ 420
  V L D S V L * L * G E A E K * Y P G A A -
GGCTTTACGTCTTCAGCCTAAACCTAAAAA AAAAAAAAAAAAAAAAAAATTTAAACAGCTA
421 -----+-----+-----+-----+-----+-----+-----+-----+ 480
  G F T S S A * N L K K K K K K N L K Q L -
TTAAACTGRAAGCATCTGTA AAAAAAAAAAAAAAAAAA
481 -----+-----+-----+-----+-----+-----+-----+-----+ 515
  L N ? K H L * K K K K -

```

Fig. 1

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SEQ ID NO: 2

The 110 aa precursor peptide preCC7 containing the 20aa search strand.

i) Sequence characteristics:

length: 110 aa
 type: amino acid
 topology: linear

ii) Molecule type: protein

```

1  MRFMTLLFLT ALACALVCAY DPEAASAPGS GNPCHEASAA QKENAGEDPG
      *      *      *      *      *      *
51  LAHQAPKPRK QSSILLEKGL DGAKKAVGGL GKLGKDAVED LESVGKGAVER
      *      *
101 DVKQVLDLVL
      *
```

MRFMTLLFLT ALACALVC: potential signal sequence
 K / R: potential basic protease cleavage site
 *: potential O-glycosylation site
 #: potential N-glycosylation site

Fig. 2

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SEQ ID NO: 17

The 24 aa cancer cachetic factor.

i) Sequence characteristics:

length: 24 aa
type: amino acid
topology: linear

ii) Molecular type: protein

1 AYDPEAASAP GSGNPCHEAS AAQK

Fig. 3a

SEQ ID NO: 18

The 20 aa cancer cachetic factor.

i) Sequence characteristics:

length: 20 aa
type: amino acid
topology: linear

ii) Molecular type: protein

1 YDPEAASAPG SGNPCHEASA

Fig. 3b

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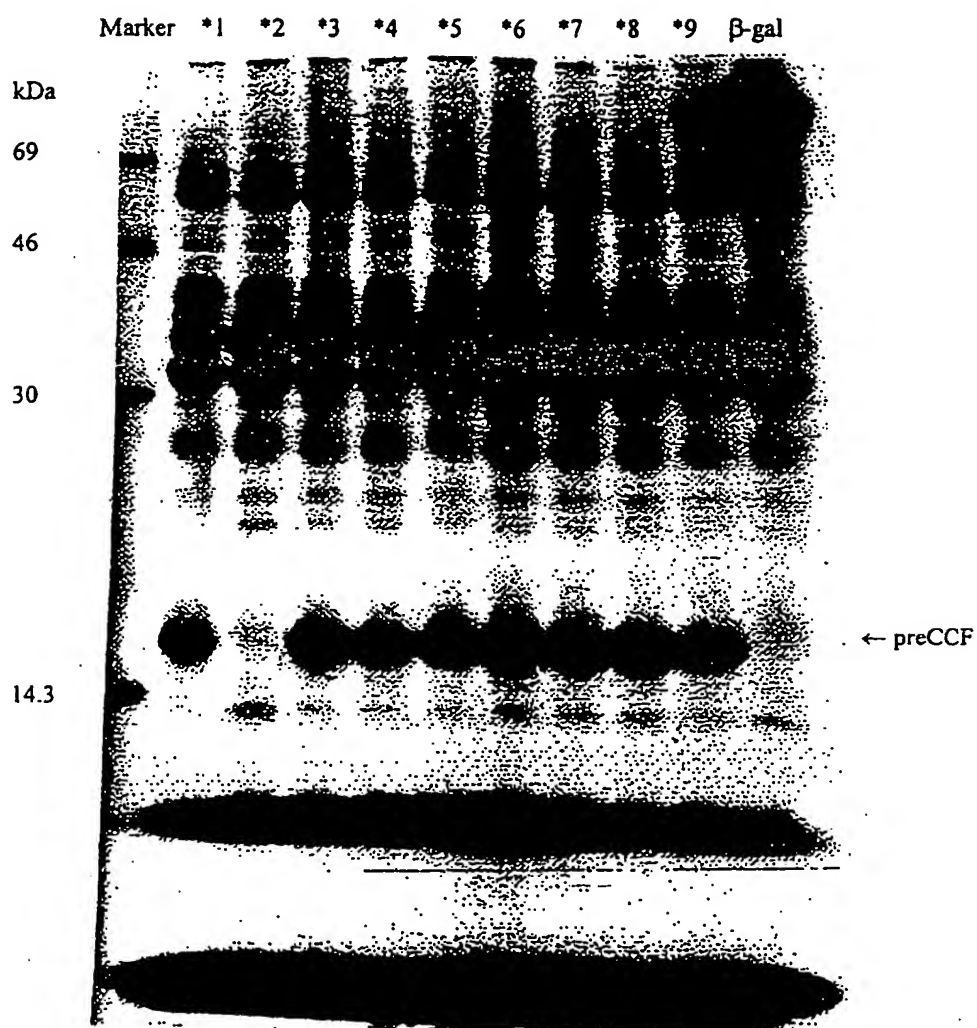


Fig. 4

1
INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00377

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/47, A61K 38/17
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

REG, CAPLUS, WPI, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E,X	WO 9738100 A1 (INCYTE PHARMACEUTICALS, INC.), 16 October 1997 (16.10.97), see seq 2 and 3	1-36,45-51, 54-55,57
A	US 5219579 A (MICHAEL J. TISDALE ET AL), 15 June 1993 (15.06.93)	1-36,45-51, 54-55,57
A	Letters to nature, Volume 379, February 1996, Penio Todorov et al, "Characterization of a cancer cachectic factor" page 739 - page 742	1-36,45-51, 54-55,57

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

- | | |
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| <p>Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> | <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> |
|--|---|

Date of the actual completion of the international search

Date of mailing of the international search report

15 December 1997

16-12-1997

Name and mailing address of the ISA/
Swedish Patent Office
Box 5055, S-102 42 STOCKHOLM
Facsimile No. +46 8 666 02 86

Authorized officer

Carolina Gómez Lagerlöf
Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 37-44, 52-53, 56
because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐
☐

- The additional search fees were accompanied by the applicant's protest.
No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/12/97

International application No.

PCT/DK 97/00377

Patent document cited in search report			Publication date	Patent family member(s)	Publication date
WO	9738100	A1	16/10/97	NONE	
US	5219579	A	15/06/93	AU 637046 B	20/05/93
				AU 3426389 A	05/10/89
				DK 222790 A	17/09/90
				EP 0335550 A	04/10/89
				GB 2217330 A,B	25/10/89
				JP 5504543 T	15/07/93
				US 5385740 A	31/01/95
				WO 8908709 A	21/09/89

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